BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT

RLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A2

(11) International Publication Number: WO 99/53097

(43) International Publication Date: 21 October 1999 (21.10.99)

(21) International Application Number: PCT/US99/07513

(22) International Filing Date: 6 April 1999 (06.04.99)

(30) Priority Data:
60/081,944 15 April 1998 (15.04.98) US

(71) Applicant *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES CENTERS FOR DISEASE CONTROL AND PREVENTION [US/US]; 255 East Paces Ferry Road, N.E. Room 500, Atlanta, GA 30333 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: KILPATRICK, David, R. [US/US]; 1095 Fulton Court, Norcross, GA 30093 (US).

(74) Agents: SAMPLES, Kenneth, H. et al.; Fitch, Even, Tabin & Flannery, Room 1600, 120 South LaSalle, Chicago, IL 60603 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD FOR DEVELOPING DEGENERATE PCR PRIMERS

(57) Abstract

The present invention provides a method for designing and making degenerate primers for use in amplification assays. Using the present method, degenerate primers for the amplification and subsequent detection of virtually all genes that encode an amino acid sequence can be obtained. The degenerate primers are effective for detection of any gene which lies within a coding region that results in the production of a protein. Examples of genes that can be detected include those where the sequence of the specific target gene is known or unknown and where the amino acid sequence encoded for by the gene is structural, nonstructural, or enzymatic. The method provides highly specific primers which are effective for substantial amplification of a target sequence even where the target nucleic acid sequence is unknown.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD FOR DEVELOPING DEGENERATE PCR PRIMERS

The present invention relates to a method for developing degenerate primers for use in amplification assays.

BACKGROUND OF THE INVENTION

A number of diagnostic assays have been developed which rely on detection of the presence of a particular DNA or RNA sequence as an indication of the presence of analyte, e.g., a bacterium, virus, or genetic defect, in a sample. In some cases the diagnostic gene is present in sufficient quantities to be detected directly, whether by hybridization, reaction with specific antibody, or by some other method. However, if the gene of interest is present in a small amount or the background caused by similar sequences present in the sample is sufficiently high, reliable and sensitive detection of the targeted gene is difficult.

The amplification of specific nucleic acid segments greatly facilitates the detection of pathogens and of disease states associated with the presence of particular nucleic acid sequences. The polymerase chain reaction (PCR) is a widely accepted and powerful tool for the enzymatic amplification of individual DNA sequences from unique oligonucleotide primers and a small amount of target DNA.

PCR involves the use of two oligonucleotide primers, an enzymatic agent for polymerization, a target nucleic acid template, and successive cycles of denaturation of nucleic acid and annealing and extension of primers to produce a large number of copies of a particular nucleic acid segment. The segment copied consists of a specific sequence of nucleosides from the target template. This specific sequence is defined by regions on the template that can hybridize to the

primers and the nucleic acid sequence between those regions. Processes for amplifying a specific nucleic acid segment by the PCR method are described in U.S. Patent Nos. 4,683,202 and 4,683,195, both of which are incorporated herein by reference.

In order for a successful PCR to occur each of the oligonucleotide primers must hybridize to sequences flanking the gene of interest. More specifically, the nucleotide sequence of the "first" primer is selected such that it is capable of hybridizing to an oligonucleotide sequence located 3' to the sequence of the desired nucleic acid molecule, whereas the nucleotide sequence of the "second" primer is selected such that it contains a nucleotide sequence identical to one present 5' to the sequence of the desired nucleic acid molecule.

One disadvantage of PCR is that it requires the use of two primers, and thus requires that sequence information be available for two regions of the target molecule. This is often a significant constraint. In some situations, only the amino acid sequence encoded by a target sequence is known. Hence, to amplify the target sequence, it is necessary to employ sets of degenerate primers (corresponding to each of the possible sequences capable of encoding the amino acid sequence coded for by the two regions of the target molecule). The use of such degenerate primer sets can cause significant priming errors, and thus an decrease amplification efficiency.

One means of decreasing the number of members in the primer sets when conducting PCR amplification is through the use of primers containing deoxyinosine at positions of ambiguity (Patil, R.V., Nucl. Acids Res. 18:3080 (1990); Fordham-Skelton, A.P. et al., Molec. Gen. Genet. 221:134-138 (1990); both of which references are herein incorporated by reference). Since dexoyinosine binds with A, T, G and C, the specificity of primers containing deoxyinosine is decreased.

A second significant disadvantage of the PCR reaction is that when two different primers are used, the reaction conditions chosen must be selected such that both primers "prime" with similar efficiency. Since the two primers necessarily have different sequences, this requirement can constrain the choice of primers and require considerable experimentation.

An object of the present invention is to provide a method of developing primers effective for use in PCR amplifications where the target nucleic acid sequence is unknown but where an amino acid sequence coded for by that target nucleic acid sequence is known.

An object of the present invention is to provide a method of developing primers effective for targeting nucleic acid sequences that allow for the correlation of the subsequent molecular based diagnosis with a serologically derived diagnosis.

Other objects, advantages, features and characteristics of the present invention will become more apparent upon consideration of the following description and the appended claims.

SUMMARY OF THE INVENTION

The method of the present invention provides degenerate primers for the amplification and subsequent detection of virtually all genes that encode an amino acid sequence. The degenerate primers are effective for detection of any gene which lies within a coding region that results in the production of a protein. Examples of genes that can be detected include those where the sequence of the specific target gene is known or unknown and where the amino acid sequence encoded for by the gene is structural, nonstructural, or enzymatic. The method provides highly specific primers which are effective for substantial amplification of a target sequence even where the target nucleic acid sequence is unknown.

The present invention improves the detection of target sites by specifying the identification of conserved adjacent amino acid residues within the coding region of two or more identical genes for diagnostic purposes. Once the amino acid target sites are found, specific oligomers are designed which recognize only that site, taking into account the degenerate coding nature of the individual amino acids. The result is the amplification of only the nucleic acids containing that specific target site, and thus related to the original sequence of adjacent amino acid residues.

The primers are designed to contain no more than about 7 degenerate positions (either dI, dK, dP, or mixed base residues). As a result, all primers function at a minimum temperature requirement of about 42°C for annealing, and about 60°C for elongation.

In an important aspect of the invention, a method is provided for making degenerate PCR primers which are effective for amplification of a target nucleic acid sequence. The method comprises identifying uniquely conserved amino acid sequences and subsequently identifying sequences of nucleic acids that corresponds to the uniquely conserved amino acid sequence. Pre-determined nucleotides are substituted at degenerate nucleotide positions such that the resulting primer has no more than about four pre-determined nucleotides and such that these pre-determined nucleotides are at least about 3 bases away from a 3' end of the primer. In addition, the degenerate PCR primer has no more than about seven degenerate positions and no more than about two adjacent pre-determined nucleotides.

In another important aspect, the present invention provides a method for amplifying a target nucleic acid. The method comprises identifying uniquely conserved amino acid sequences and subsequently identifying sequences of nucleic acids that corresponds to the uniquely conserved amino acid sequence. Pre-determined nucleotides are substituted at degenerate nucleotide positions such that the resulting primer has no more than about four pre-determined nucleotides and such that these pre-determined nucleotides are at least about 3 bases away from a 3'

end of the primer. In addition, the degenerate PCR primer has no more than about seven degenerate positions and no more than about seven degenerate positions and no more than about two adjacent pre-determined nucleotides. The degenerate PCR primers are added to a PCR assay in an amount effective for amplifying any target nucleic acid present in the sample and PCR is conducted at a temperature effective for amplifying target nucleic acid. The presence of amplified target nucleic acid is detected using methods known in the art.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS. The term "primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleoside triphosphatase and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligodeoxynucleotide. The appropriate length of a primer depends on the intended use of the primer but typically ranges from about 17 to about 23 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template nucleic acid, but must be sufficiently complementary to hybridize with the template. Primers can incorporate additional features which allow for the detection or immobilization of the primer but do not alter the basic property of the primer (i.e., acting as a point of initiation of DNA synthesis). For example, primers may contain an additional nucleic acid sequence at the 5' end which does not hybridize to the target nucleic acid, but which facilitates cloning of the amplified product. The region of the primer which is sufficiently complementary to the template to hybridize is referred to herein as the "hybridizing region".

Oligonucleotides can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical

synthesis by a method such as the phosphotriester method of Narang et al., Meth. Enzymol. 68:90-99 (1979); the phosphodiester method of Brown et al., Meth. Enzymol. 68:109-151 (1979); the diethylphosphoramidite method of Beaucage et al., Tetrahedron Lett. 22:1859-1862 (1981); and the solid support method of U.S. Pat. No. 4,458,066, each incorporated herein by reference. A review of synthesis methods is provided in Goodchild, Bioconjugate Chemistry 1(3):165-187 (1990), incorporated herein by reference.

"Amplification" as used herein refers to an increase in the amount of the desired nucleic acid molecule present in a sample. "Substantial amplification" refers to greater than about three-fold amplification. The primers developed according to the method of the present invention are effective for providing at least about 1 million fold amplification of a target nucleic acid, assuming a minimum of about 30 PCR amplification cycles.

The term "hybridization" refers the formation of a duplex structure by two single-stranded nucleic acids due to complementary base pairing. Hybridization can occur between fully complementary nucleic acid strands or between "substantially complementary" nucleic acid strands that contain minor regions of mismatch. Conditions under which only fully complementary nucleic acid strands will hybridize are referred to as "stringent hybridization conditions" or "sequence-specific hybridization conditions". Stable duplexes of substantially complementary sequences can be achieved under less stringent hybridization conditions. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length and base pair concentration of the oligonucleotides, ionic strength, and incidence of mismatched base pairs, following the guidance provided by the art (see, e.g., Sambrook et al., Molecular Cloning-A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1985), Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wilty-Interscience, John Wiley and Sons, N.Y. (1987 updated quarterly) (each incorporated herein by reference).

DNA typically contains a polynucleotide composed of the 4 "natural" bases: A(adenine), T (thymine), C (cytosine), and G (guanine). The hydrogen bonding (or base pairing) among these nucleotides creates the double-stranded structure of a DNA molecule. An A-containing residue base pairs to a T-containing residue through the formation of two hydrogen bonds; a G-containing residue base pairs to a C-containing residue through the formation of three hydrogen bonds.

The term "pre-determined nucleotides" is intended to refer to nucleotides which have bases other than the A, T, C or G naturally found in DNA. Although the pre-determined nucleotides will be capable of hydrogen bonding with naturally occurring nucleotides (such as the A, T, C or G-containing nucleotides of the template), it will form fewer hydrogen bonds with such nucleotides than would other naturally occurring nucleotides.

A nucleotide containing deoxyinosine ("dI") is a preferred example of a such a pre-determined nucleotide containing the base, inosine. It is capable of forming two hydrogen bonds with either A, C, T, or G (Barker, R., Organic Chemistry of Biological Molecules, Prentice-Hall, N.J. (1971)). Thus, in an important aspect of the invention, when I is used in a primer in lieu of G or, in lieu of C, the base pairing efficiency is altered.

Other examples of "pre-determined nucleotides are those which contain, 2-amino-6-methoxyaminopurine (=k, a purine analog which can replace A and G residues), and 6H, 8H-3, 4-dihydropyrimidol [4,5-c] [1,2] oxazin-7-one (=P, a pyrimidine analog which can replace T and C residues), with these base analogs being known as dK and dP, respectively (Lin and Brown, 1994. Chapter 7: Oligonucleotides Containing Degenerate Bases. in Methods in Molecular Biology, Vol 26: Protocols for Oligonucleotide Conjugates, Edited by S. Agrawal, Humana Press Inc., Totowa, NJ.). These base analogs may be used in order to further reduce the number of possible primer "species" within a primer by replacing a base mixture. For instance, the base mixture A/G could be replaced with the single base, dK, while the base mixture T/C could be replaced with the single base, dP.

The terms "target region" and "target nucleic acid" refers to a region of a nucleic acid which is to be amplified, detected, or otherwise analyzed. Target nucleic acid capable of being amplified through use of the primers developed by the methods of the present invention includes any prokaryotic or eukaryotic nucleic acid (DNA or RNA), which can include any plant, animal, bacterial, viral, fungal, etc., nucleic acids for which at least one nucleic acid sequence is known. The sequence to which a primer or probe hybridizes can be referred to as a "target sequence".

As used herein, "uniquely conserved" means an amino acid epitope (i.e. linear stretch of adjacent amino acids) which is found to be identical in 2 or more genes, or parts of genes, and not found in other gene sequence within that specific organism, or between organisms.

As used herein the term "primer species" means the total number of oligomers present after the synthesis of a primer, for which the number is equal to 2^n, where (n) represents the number of positions within the primer containing 2 base mixtures.

SELECTION OF PCR PRIMER TARGET SITES

1. Identify six to seven uniquely conserved linear stretches of amino acids as PCR primer target sites.

A conserved six to seven amino acid target is required in order to specify 17-21 nucleotides, which is the optimal PCR primer size. Degenerate PCR primers used as diagnostic PCR primers should, in general, target structural proteins due to the conservation of structural motifs (i.e. those sites involved in protein folding, intercellular transport, processing, expression, etc.) and relatedness to serology (i.e. antibody neutralization sites, major histocompatibility binding sites, peptide processing sites for immunological presentation, etc.). Providing PCR primer which target structural proteins allows for a clinical diagnosis of infections which correlates a molecular based diagnosis with a serologically derived diagnosis. In

an important aspect of the invention, the targeting of immunological sites via degenerate PCR primers allows an assay combining both molecular and immunological methods with one reagent.

Example 2 listed below describes the use of degenerate PCR primers for the identification of poliovirus, serotypes 1, 2 and 3. The serotype 1 specific PCR primer targets a known MHC I T-cell helper epitope (Kutubuddin et al., 1992), while the serotype 3 specific PCR primer targets the known poliovirus neutralization site 2a (Minor, 1990).

Degenerate PCR primers may also target proteins which have non-structural functions and which have a broader relationship between organisms or groups of related organisms. For example, important targets may include a plethora of enzymes with specific functions involving replication, transcription, translation, morphogenesis, protein/nucleic acid modifications, etc. A particle list includes enzymes such as DNA or RNA polymerases, nucleuses (both RNAses and DNAses), protein kinases, neuraminidases, ribonuclease H, ATPases, etc.

In an important aspect of the invention, when targeting double-stranded DNA sequences, the targeted amino acid site should be absolutely unique and conserved in both strands of DNA. For this reason, the alternate DNA strand should be searched for possible non-specific amplification due to the degenerate nature of the primer.

The amplification reactions with RNA's, (most of which are single stranded with the exception of reoviruses which contain double-stranded RNA), either 1 primer or the other specifically hybridizes to the RNA. Both primers do not initially anneal to the nucleic acid since there is only "1" strand present. However, DNA (most of which is double-stranded, except for few single DNA strand containing viruses, ie. parvoviruses) presents a different problem since both PCR primers could hybridize, 1 to each specific strand of DNA, and thus initiate possibly "different" template amplifications. This is the reason that the target

region has to be more specific (ie. the gene should be "searched" if possible for the ability of "both" oligomers being able to anneal to the nucleic acid), due to the possibility of the "second" PCR primer being able to anneal to a DNA sequence.

In an another important aspect of the invention, the amino acid target sites should be of a size effective for providing degenerate PCR primers which are about 17 to about 23 nucleotides in length. Oligomer which are smaller than 17 nucleotides or larger than 23 nucleotides are less effective as they loose specificity due to the degeneracy of the primer. In a preferred embodiment, the degenerate PCR primer is about 20 nucleotides in length.

2. Determine sequences of nucleotides that correspond to the uniquely conserved amino acid sequences.

Once a uniquely conserved amino acid sequence has been identified, all nucleic acid sequences which may code for that amino acid sequence can then be identified by reference to the genetic code.

3. Substitute the base analog deoxyinosine at positions within the PCR primer where 3 or 4 nucleotides could be used.

Due to the degeneracy of the genetic code, there are a number of amino acids which are coded for by different combinations of nucleotides. For example, the 3rd position of isoleucine can be T(U), C, or A. Hence, in providing degenerate PCR primers, the 3rd position of codons which codes for isoleucine are substituted with a pre-determined nucleotide. Further, the third position of codons which code for alanine, arginine, glycine, leucine, proline, serine, threonine, and valine can be any one of four nucleotides. Hence, in providing degenerate PCR primer, the 3rd position of codons which code for these amino acids is substituted with a pre-determined nucleotide. In an important aspect of the invention, the pre-determined nucleotide used for substitution is deoxyinosine, but could also include dK and dP.

CONSIDERATIONS IN SELECTING AN AMINO ACID TARGET SITE

1. Select amino acid target sites effective for positioning a pre-determined nucleotide residue at least about 3 bases away from the 3' end of the PCR primer, and preferably about 5 or more bases from the 3' end of the PCR primer.

During PCR amplification, an appropriate enzyme (e.g., a Taq polymerase) is added which begins its synthesis of adding the next corresponding nucleotide at the 3' end of the PCR primer. Since, a pre-determined nucleotide such as dI, dK, and dP bond more weakly to individual nucleotides, primers having dI, dK or dP less than about 3 bases away from the 3' end of the primer yield weaker amplifications or no amplifications at all. Hence, it is preferable to keep codons which code for the most degenerate amino acids towards the 5' end or middle of the PCR primer. The order of amino acids for targeted sites from least degenerate to most degenerate is (MET, TRP) > (CYS, TYR, PHE, GLN, HIS, ASP, GLU, ASN, LYS) > ILE > (PRO, GLY, VAL, ALA, THR) > (SER, LEU, ARG).

2. Select amino acid target sites effective for providing a PCR primer with no more than about 7 degenerate positions.

In order to maintain the specificity of the PCR primers, it is preferable to have a maximum of about 7 degenerate position within a 20 nucleotide base PCR primer. Of these 7 degenerate positions, no more than 4 of these positions should include either dI, dK, dP, or mixed base residues. For example, a primer could contain either 4 deoxyinosine residues and 3 mixed base residues, or 4 mixed base residues and 3 deoxyinosine residues. Having fewer degenerate positions within a PCR primer increases the specificity.

3. PCR primers should have no more than about 16 primer species within any one primer.

In an important aspect of the invention, the primer contains no more than about 4 mixed base residues. To further reduce the primer species, the base analogs dK and dP can be substituted at primer sites which call for either A/G mixtures or T/C mixtures, respectively. In another aspect of the invention, a

deoxyinosine residue may be used even where only 2 different nucleotides are possible in a particular position. This reduces the number of mixed base positions, reduces the number of primer species, and increases the specificity of the PCR primer.

4. Select amino acid target sites effective for providing primers that have no more than abut 2 adjacent deoxyinosine residues.

If three or more adjacent deoxyinosine residues are needed, it is an indication that the targeted site is not completely conserved. Primers developed from target sites which are not completely conserved limit the specificity of the primer and may lead to false positive PCR reactions or no amplification at all, both of which are unacceptable for diagnostic and detection purposes.

5. Primers should yield a PCR product having less than about 150 base pairs in size.

To reduce the chances of non-specific PCR products, PCR primer sets should yield the smallest PCR products possible, preferably less than about 150 base pairs (Yang et al., 1991; Kilpatrick et al. 1996).

USE OF DEGENERATE PCR PRIMERS IN PCR REACTIONS

Primers developed according to the method of the present invention are added to a PCR assay in an amount effective for amplifying any target nucleic acid present in the sample. In an important aspect of the invention, at least about 5 picomoles (pmoles) of each primer species should be used in a PCR reaction. It is considered "state of the art" to use between 5 and 10 pmoles of primer per 50 ul PCR reaction, for optimum amplification (Perkin Elmer). At the minimum of 5 pmoles per species, a primer with 4 mixed base positions would have 5pm X 16 species, for a total of 80 pm. If both primers in the PCR reaction have 4 mixed positions, there would be a total of 2 X 80 pm, for a total of 160 pm of primers in a 50 microliter reaction. Assuming that each primer was 20 bases in length, then this would be equal to 1 microgram of total primer per reaction (160pm X 20 X

325/1,000,000; Perkin Elmer catalog). It becomes impractical (and very expensive) to use higher quantities of synthesized primers per reaction.

In another important aspect of the invention, the PCR should be conducted at a temperature of about 95°C for the denaturation step, about 42°C for the annealing step, and about 60°C for the extension step. The 42°C annealing temperature is important in order to allow the degenerate primers to successfully anneal because degenerate primers have a lower melting temperature (well below 50°C, in some cases) due to the base analogs dI, dK, and dP. Higher temperatures may result in either poor or lack of amplification due to the primers "melting" off of the template when the anneal and extension temperatures are raised. However, in some cases (as determined by experiment) these temperatures can be increased without impeding the amplification reaction.

The PCR reaction using the primers developed by the methods of the present invention should be conducted in a detergent free buffer. As used herein, the term "detergent free buffer" means a buffer having a concentration of 0% detergent, such as Triton X-100. This was determined by the failure of a PCR buffer containing Triton X-100. Other similar ionic detergents may have the same affect. The PCR buffer composition is not critical as long as the buffer is detergent free.

PCR and the resulting detection of PCR products are accomplished by methods known in the art.

The use of degenerate PCR primers developed by the method of the present invention is useful in PCR screening assays to speed the identification of samples by reducing the number of subsequent assays (such as immunoassays or neutralization assays) that need to be performed to verify the identity of the sample. PCR using degenerate primers developed according to the method of the present invention can, for example, detect as little as about 100 fg of viral RNA.

The following examples illustrate methods for carrying out the invention and should be understood to be illustrative of, but not limiting upon, the scope of the invention which is defined in the appended claims.

EXAMPLES

EXAMPLE 1: Development and Use of Degenerate Primers for the Detection of Poliovirus.

An example of the use of the method of the present invention to develop poliovirus specific primers, and use of those primers for the detection of polioviruses in clinical samples is provided in U.S. Patent No. 5,585,477, issued December 17, 1996, which is incorporated herein by reference.

EXAMPLE 2: Development and Use of Degenerate Primers for the Serotype Differentiation of Polioviruses

An example of the use of the method of the present invention to develop serotype-specific poliovirus primers is provided in the U.S. application No. 08/273,474, to which is incorporated herein by references.

EXAMPLE 3: PCR Detection of Non-Polio Enteroviruses Using Primers Containing Mixed Base or Deoxyinosine Residues at Positions of Codon Degeneracy.

Viruses: Enterovirus isolates were identified by neutralization pools of immune sera (Melnick, Virology, 2nd Ed., Raven Press, N.Y., 1990) followed by confirmation of serotype with monotype neutralizing polyclonal antibodies. Viruses were propagated in HeLa or RD monolayers to produce high-titer inoculation stocks.

Amino acid sequences: All of the VP1 amino acid sequences in Table 1 were obtained from Genbank.

TABLE 1: NPEV VP1 AMINO ACID ALIGNMENT WITHIN THE VP1 PROTEINS OF 15 HUMAN ENTEROVIRUS STRATINS

A) Amino acid positions 1-80

```
CAV24   GIEETIDTVISNALQLSQPKPQKQLTAQSTPSTS....GVNSQEVPALTAVETGVSGQAIPSDVIETRHVVNYKTRSESTL
CAV21   GIEDLIDTAIKNALRVSQPLRPSQLKQ.........PNGVNSQEVPALTAVETGASGQAIPSDVVETRHVINYKTRSESCL
CAV16   GDGIADMIDQAVTSRVGRALTSLQVEPTAANTNASE.HRLGTGLVPALQAAETGASSNAQDENLIETRCVLNHHSTQETTI
CAV2    GDGIEDAITNTVNATINRVLDRPISHSSTAANTQVSQHSIETGRVPALQAAETGATSNASDENLIETRCVVNKNSVEEASL
CAV9    GDVEEAIERARCTVADTMRTGPS..................NSASVPALTAVETGHTSQVTPSDTMQTRHVKNYHSRSESTV
CBV1    GPVEESVERAMVRVADTVSSKPT..................NSESIPALTAAETGHTSQVVPSDTMQTRHVKNYHSRSESSI
CBV3    GPVEDAITAAIGRVADTVGTGPT..................NSEAIPALTAAETGHTSQVVPGDTMQTRHVKNYHSRSESTI
CBV4    GPTEESVERAMGRVADTIARGPS..................NSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYHSRSESSI
CBV5    GPPGEAVERAIARVADTISSGPV..................NSESIPALTAAETGHTSQVVPADTMQTRHVKNYHSRSESTY
Echo6   NDVQNAVERSIVRVADTLPSGPS..................NSESIPALTAAETGHTSQVVPSDTIQTRHVRNFHVRSESSV
Echo9   SDVREAVEGAIGRVADTIRSGPS..................NSEAVPALTAAETGHTSQVVPSDTMQTRHVKNYHSRSESTI
Echo11  GDVVEAVENAVARVADTIGSGPS..................NSQAVPALTAVETGHTSQVTPSDTMQTRHVKNYHSRSESSI
Echo12  GDVEEAVNRAVARVADTLPTGPR..................NSESIPALTAAETGHTSQVVPGDTMQTRHVKNYHSRTESSV
EV70    GEIVKTVANTVE.......................SEIKAELGVIPSLNAVETGATSNTEPEEAIQTRTVINMHGTAECLV
EV71    GDRVADVIESSIGDSVSRALTQALPAPTGQNTQVS.SHRLDTGEVPALQAAEIGASSNTSDESMIETRCVLNSHSTAETTL
```

B) Amino acid positions 81-163

```
CAV24   ESFFGRSACVTMLEVENFNATTEADKKKQFTTWAITYTDTVQL.RRKLEFFTYSRFDLEMTFVITERYYTSNT..GYARNQVYQ
CAV21   ESFFGRAACVTILSLTNSSKSGEE..KKHFNIWNITYTDTVQL.RRKLEFFTYSRFDLEMTFVFTENYPSTAS..GEVRNQCDQ
CAV16   GNFFSRAGLVSIITMPTTGTQNTDGY....VNWDIDLMGYAQM.RRKCELFTYMRFDAEFTFVAAKPN.......GELVPQLLQ
CAV2    NHFFSRAALVGKVELNDTGTAATGFT....NWNIDIMGYAQL.RRKLEMFTYMRFNAEFTFVATTRA........GRVPSRVLQ
CAV9    ENFLGRSACVYMEEYKTTDKHVN...KKF.VAWPINTKQMVQM.RRKLEMFTYLRFDMEVTFVITSRQDPGTTLAQDMPVLTRQ
CBV1    ENFLCRSACVYYATYNNNSEKG...YAE....WVINTRQVAQLLRRKLEF.TYLRFDLELTFVITSAQEPSTATSVDAPVQTQQ
CBV3    ENFLCRSACVYFTEYKNSGAKR...YAE....WVLTRRQAAQL.RRKLEFFTYVRFDLELTFVITSTQQPSTTQNQDAQILTHQ
CBV4    ENFLCRSACVIYIKYSSAESNNLKRYAE....WVINTRQVAQL.RRKMEMFTYIRCDMELTFVITSHQEMSTATNSDVPVQTHQ
CBV5    ENFLCRSACVYYTTYKNHGTDGNFAY......WVINTRQVAQL.RRKLEMFTYARFDLELTFVITSTQEQSTIQGQDSPVLTHQ
Echo6   ENFLSRSACVYIVEYKTQDTTPD....KMYDSWVINTRQVAQL.RRKLEFFTYVRFDVEVTFVITSVQDDSTRQNTDTPVLTHQ
Echo9   ENFLCRSACVRMAKYEARGNLKA....LTLDAWEISVRDMVQL.RRKCEMFTYLRFDVEVTFVITSYQRQGTSSIQICPYDAHQ
Echo11  ENFLSRSACVYMGGYHTTNTDQT....KLFASWTISARRMVQM.RRKLEIFTYVRFDVEVTFVITSKQDQGSRLGQDMPPLTHQ
Echo12  ENFLCRAACVCITKYKTKDSDPV....QRYANWRINTRQMAQL.RRKFELFTYLRFDMEVTFVITSSQDDGTQLAQDMPVLTHQ
EV70    ENFLGRSALVCMRSFEYKNHSTSTSSIQKNFFVWTLNTRELVQIRRKMELFTYLRFDTEITIVPTLRLFSSSNASSSGLPNLTLQ
EV71    DSSFFSRAGLVGEIDLPL...EGTTNPNGYANWDIDITGYAQ.MRRKVELFTYMRFDAEFTFV.......ACTPTGEVVP.QLLQ
```

C) Amino acid position 164-241

```
CAV24    LMYIPPGAPRPTAWDDYTSQSSSNPSVFYTYGSAPPRISIPYVGIANAYSHFYDGFARVPLKDETVDSGDTYYGLVTI
CAV21    IMYIPPGAPRPSSWDDYTWQSSSNPSIFYMYGNAPPRMSIPYVGIANAYSHFYDGFARVPLEGENTDAGDTFYGLVSI
CAV16    YMYVPPGAPKPTSRDSFAWQTATNPSIFVKLTDPPAQVSVPFMSPASAYQWFYDGYPTFGAHPQSNDADYGQCP....
CAV2     YMYVPPGAPKPDGREAFQWQSSTNPSVFSKMTDPPPQVSVPFMSPASAYHGFYDGYPTFGEHNGEDSLRTGNA.....
CAV9     IMYVPPGGPIPAKVDDYAWQTSTNPSIFWTEGNAPARMSIPFISIGNAYSNFYDGWSNFDQRGSYGYNTL........
CBV1     IMYVPPGGPVPTKVTDYAWQTSTNPSVFWTEGNAPPRMSIPFISIGNAYSCFYDGWTQFSRNGVYGINTL........
CBV3     IMYVPPGGPVPDKVDSYVWQTSTNPSVFWTEGNAPPRMSIPFLSIGNAYSNFYDGWSEFSRNGVYGINTL........
CBV4     IMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNAPPRMSIPFMSIGNAYTMFYDGWSNFSRDGIYGYNSL........
CBV5     IMYVPPGGPVPTKINSYSWQTSTNPSVFWTEGSAPPRISIPFISIGNAYSMFYDGWAKFDKQGTYGINTL........
Echo6    IMYVPPGGPIPHAVDDYNWQTSTNPSVFWTEGNAPPRMSIPFMSVGNAYSNFYDGWSHFSQTGVYGFNTL........
Echo9    IMYIPPGGPIPKKVDGYEWQTSTNPSIFWTEGNAPPRMSIPFISIGNAYSSFYDGWSHFDSKGAYGFNTL.........
Echo11   IMYIPPGGPIPKSVTDYAWQTSTNPSIFWTEGNAPPRMSIPFISIGNAYSNFYDGWSHFSQNGVYGYNTL.........
Echo12   VMYIPPGGPVPNSATDFAWQSSTNPSIFWTEGNAPARMSIPFISIGNAYSNFYDGWSHFTQDGVYGFNSL.........
EV70     VMYVPTGAPKPSSQDSFEWQSACNPSVFFKINDPPARLTIPFMSINSAYANFYDGFAGFEKKAT..DL....YGINPA
EV71     YMFVPPGAPKPESRESLAWQTATNPSVFVKLTDPPAQVSVPFMSPASAYQWFYDGYPTFGEHKQEKDLE...YGACP.
```

D) Amino acid positions 242-330

```
CAV24    NDFGTLAVRVVNEFNPARIISKIRVYMKPKHVRCWCPRPPRAVPY.RGEGVDFKQDSITPLIAVEN.INTF.............
CAV21    NDFGVLAVRAVNRSNPHTIHTSVRVYMKPKHIRCWCPRPPRAVLY.RGEGVDMISSAIQPLTKVDS.ITTF.............
CAV16    NNMMGTFSIRTVGTEKSPHSITLRVYMRIKHVRAWIPRPLRNQPYLFKTNPNYKGNDIKCTSTSRDKITTL.............
CAV2     NNALGTFSVRFVSEEITNERIIIRIYMRLKHIRAWVPRPLRSEPYVLKNFPNYTAVTHVTANRPSITNTGRF.............
CAV9     NNLFGIYVRHVSGSSPHPITSTIRVYFKPKHTRAWVPRPPRLCQYKKAFSVDFTPTPITDTRKD...
         INTVAQSRRRGDMSTLNTHGAF
CBV1     NNMGTLYMRHVNEAGQGPIKSTVRIYFKPKHVKAWVPRPPRLCQYEKQKNVNFNPTGVTTTRSN...ITTT.............
CBV3     NNMGTLYARHVNAGSTGPIKSTIRIYFKPKHVKAWIPRPPRLCQYEKAKNVNFQPSGVTTTRQS...ITTM.............
CBV4     NNMGTIYARHVNDSSPGGLTSTIRIYFKPKHVKAYVPRPPRLCQYKKAKSVNFDVEAVTAERAS...LITT.............
CBV5     NNMGTLYMRHVNDGSPGPIVSTVRIYFKPKHVKTWVPRPPRLCQYQKAGNVNFEPTGVTESRTE...ITAM.............
Echo6    NNMGKLYFRHVNDRTISPITSKVRIYFKPKHVKAWVPRPPRLCEYTHKDNVDYEPKGVTTSRTS...ITITNSKHMETHGAF.....
Echo9    NKMGHIYCRHVNKETPTKVTSYIRIYFKPKHVRAWVPRPPRLCQYMNKANVNFEATAVTDTRDT...INTVPLSTHGVSRGAY....
Echo11   NHMGQIYVRHVNGSSPLPMTSTVRMYFKPKHVKAWVPRPPRLCQYKNASTVNFTPTNVTDKRTS...INYIPE.............
Echo12   NNMGSIYIRHVNEQSPYAITSTVRVYFKPKHVRAWVPRPPRLCAYEKSSNVNFKPTDVTTSRTS...ITEVPS.............
EV70     NTMGNLCLRVVNSYQPVQYTLTVRVYMKPKHIKAWAPRAPRTMPYTNILNNNYVGRSAAPNAPTAIVSDRSTIKTMPN
         DIDLTTA...
EV71     NNMMGTFSVRTVGSSKSKYPLVVRIYMRMKHVRAWIPRPMRNQNYLFKANPNYTGNSIKPTGTSR..........NAITTL...
```

Abbreviations for virus groups are followed by serotype number:

CAV: Coxsackievirus A

CBV: Coxsackievirus B

Echo: Echovirus

EV: Enterovirus

Their accession numbers are as follows: CAV2-L28146, CAV9-D00627, CAV16-U05876, CAV21-D00538, CAV24-D90457, CBV1-M16560, CBV3-M33854, CBV4-X05690, CBV5-X67706, EV70-D00820, #V71-U22521, Echo 6-U05851, Echo 9-X84981, Echo 11-X80059, Echo 12-X77708.

Oligonucleotide synthesis:

Synthetic Oligodeoxynucleotides were prepared, purified, and analyzed as described (Yang et al., Virus Res. 20: 159-179, 1991). The degenerate primers used for virus amplification are listed in Table 2.

Table 2: NPEV PCR PRIMERS

PRIMER*	TARGETED SEQUENCE**	DEGENERATE PRIMER SEQUENCE***
1A	FGQQSGA (3-9)2	5'-GCICCIGAYTGITGICCRAA
5S	MYVPPGG (142-148)1	5'-ATGTAYGTICCICCIGGIGG
6A	WTEGNAP (169-175)1	5'-GGIGCRTTICCYTCIGTCCA
6S	WTEGNAP (169-175)1	5'-TGGACIGARGGIAAYGCICC
7A	N(ts)LNNM (208-213)1	5'-CATRTTRTTIARIGWITT
7S	N(ts)LNNM (208-213)1	5'-AAIWCIYTIAAYAAYATG
8A	GATG(yq)QS (1-7)2	5'-GATTGSTIICCRAAIGCKCC
9A	FKPKHVK (237-243)1	5'-TTIACRTGYTTIGGYTTRAA
11A	TMQTRHV (47-53)1	5'-ACRTGICIIGTYTGCATIGT
14S	A(mi)(gv)RVAD(10-16)1	5'-GCIATIGKIMGIGTIGCIGA
24S	PALTA(av)E (42-48)1	5'-CCIGCICTYACTGCIGYKG
25A	NY(kh)(st)RSE(63-69)3	5'-TCAGAICIIGWITKRTARTT
27A	PALTAVE (42-48)1	5'-TCCACIGCAGTIAGWGCWGG
28A	GEVRNQ (143-148)3	5'-CARGTICGIACYTCCCC
34S	FTYVRFD (107-113)4	5'-TTIACITAYGTICGITTYGA
35A	PVQT(hq)QI(135-141)1	5'-ATYTGITGIGTYTGIACWGG
36S	ELTFVIT (115-121)1	5'-GARYTIACITTYGTIATAAC
38A	MPVLTRQ (73-79)5	5'-TGICGIGTYAAIACIGGCAT
39S	FTYMRFD (107-113)6	5'-TTIACITAYATGCGITTYGA
40A	NGELVPQ (63-69)6	5'-TGIGGIACIAGYTCICCRTT
41A	CTPTGRV (60-66)7	5'-ACYCTICCIGTIGGKGTRCA
43A	MY(vi)P(tp)GA(153-159)3	5'-GCICCIGKIGGIAYRTACAT
46A	NYHSRSE (55-61)1	5'-TCIGAICTIGWRTGRTARTT
47A	MQTRHV(kh) (48-54)1	5'-TKIACRTGICKIGTYTGCAT
51S	(cnm)FYDGW(191-196)1	5'-AWITTYTAYGAYGGITGG
52A	NNMGT(il)Y(211-217)1	5'-TAIAIIGTICCCATRTTRTT
54A	NNNYVGQ (255-261)8	5'-TGICCIACRTAITTRTTRTT
55S	VVNSYQP (215-221)8	5'-GTIGTIAAYTSITAYCARCC
59S	GDGIADM (1-7)6	5'-GGIGAYGGIATIGCIGAYATG
61S	MYVPGGA (153-159)3	5'-ATGTAYRTICCIMCIGGIGC
63S	ITERYYT (140-146)9	5'-ATIACIGARIGITAYTAYAC
64S	DENLIET (60-66)6	5'-GAIGARAAYCTIATIGARAC
65A	WDID(il)(mt)G(109-115)6	5'-CCCATIAKRTCIATRTCCC
67S	KHV(rk)AWV(140-146)1	5'-AARCAYGTIARIGCITGGGT
68A	K(lm)TDPPP(182-188)6	5'-GGIGGIGGRTCIGTIAKYTT
69A	MGYAQ(ml)R(114-120)6	5'-CGIAKYTGIGCRTAICCCAT
73A	D(tm)PVLTH (136-142)10	5'-TGIGTIAGIACIGGCRTRTC

* A=antisense, S=sense

** All amino acid residues (with corresponding position numbers are located in VP1, with the exception of #2-which are located in the 2A nonstructural protein and are from the following isolates: 1=CBV-B1; 2=CBV-B1; 3=CAV-A21; 4=CBV-B3; 5=CAV-A9; 6=CAV-A16, 7=EV 71; 8=EV 70; 9=CAV-A24; 10=echovirus 12

***(Mixed base residues are as follows: Y=both T & C; R=A & G; M=A & C; K=G & T; S=G & C; W=A & T; I=deoxyinosine

Each NPEV, whose amino acid sequence in Table 1 provided the source of the targeted amino acids, is identified along with the numbers in parentheses indicating the genomic intervals matching these amino acids.

PCR amplification and analysis:

Amplification reactions were carried out in 50 ul reaction mixtures containing 1 ul of each individual virus tissue culture lysate in 50 mM Tris-HCl (ph 8.3), 70 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 80 pmol of each degenerate primer, 200 uM each of d ATP, dCTP, dGTP, dTTP (Pharmacia), 0.5% NP-40, 5 U placenta ribonuclease inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1.25 U AMV reverse transcriptase (Boehringer Mannheim), and 1.25 U of Taq DNA polymerase (Perkin Elmer-Cetus, Norwalk, CT). The reaction mixtures were prepared, excluding the ribonuclease inhibitor, AMV reverse transcriptase, and Taq DNA polymerase, overlaid with mineral oil, heated for 5 min at 95°C to release the virion RNA and chilled on ice. The enzymes were then added and the samples incubated at 42°C for 30 min for 30 cycles of programmed amplification (denaturation: 94°C, 1 min; annealing: 42°C, 1 min; extension: 60°C, 1 min) in a DNA thermal cycler (Perkin Elmer-Cetus). Conditions for polyacrylamide gel electrophoresis, and detection of amplified products by ethidium bromide staining were as described by Yang et al., Virus Res. 20:159-179 (1991).

Selection of primer binding sites.

The VP1 sequence information for 15 protype NPEVs is shown in Table 1. VP1 sequence for the remaining 50 NPEVs have not been determined. It was unknown whether amino acid sequences in these 15 prototype VP1 genes would show any conservation unique to either different groups or to specific serotypes of NPEVs. However, since the immune system recognizes enterovirus serotypes differently (as evidenced by their specific identification using the Lim and Benyesh-Melnick (LBM) pools of antisera), it was considered reasonable that conserved amino acid sequences may play some role in eliciting serotype or group-specific recognition.

Several areas of amino acid conservation can be identified in Table 1. One example of such conservation is shown by the degenerate primer pair 5S/6A. The sense primer 5S targets the amino acids MYVPPGG (a.a. #142-148 in CBV1, for example). This amino acid sequence is highly conserved in all known NPEVs. In addition, (Palmenburg, Sequences of Picornavirus Capsid Proteins, ASM, Washington, D.C. (1989)), showed this amino acid sequence is highly conserved among polioviruses and rhinoviruses as well. The PCR primer 6A is the selective primer in the amplification reaction and recognizes the amino acids WTEGNAP (a.a. #169-175 in CBV1, for example). This primer is uniquely conserved primarily among CBVs and those NPEVs closely related to CBVs, such as CAV9, and all four sequenced echoviruses (Table 1). The fact that the 5S/6A primer set also appears to recognize CAV9 is not unexpected. Other researchers have shown, using sequence alignment programs, that CAV9 is very closely related to CBVs (Pulli et al., Virology, (1995)). Many other conserved amino acid epitopes (6-7 residues in length) were identified and all are listed in Table 2 along with their relative position within VP1 and their corresponding degenerate PCR primer. To complement all possible codon combinations for all of the selected primer cited, these PCR primers contain either mixed-base residues or deoxyinosine residues at degenerate codon positions. Deoxyinosine residues, which can pair with all four bases (Martin et al., Nucleic Acids Res. 13: 8927-8938 (1985)); Ohtsuka et al., Journal Biol. Chem. 260:2605-2608 (1985)); Rossolini et al ., Mol: Cell Probes 8: 91-98 (1994)) were incorporated into the primers to match those positions having possible four-fold degeneracy. Some primers were designed to recognize more than 1 amino acid at a particular residue and are indicated by parentheses around that residue in Table 2. For example, primer 7S-N (ts) LNNM, was designed to recognize nucleotides which code for either a (T) residue (found in CBV1) or an (S) residue (found in CBV4) in the second amino acid position of the primer. As a result, the synthesis of this primer results in several species of primers with one-half of the primer species containing TGI residues (which encodes for Serine) and the other one-half containing AGI residues (which encodes for Threonine) in positions 4, 5, 6, respectively. Some of the other group-specific amino acid

epitopes that were targeted include 51S/52A, which recognizes all known CBV isolates. In this case, the sense primer 51S is the selective primer in the amplication reaction with the "FYDGW" amino acid sequence being specific for CBVs and closely related serotypes such as many of the echoviruses, while excluding CAVs from amplification. On the other hand, primers 61S/68A and 64S/65A were designed using CAV16 known sequences to target CAVs in general.

Amplification of enterovirus templates.

In order to determine if all of the isolates tested in this report contain amplifiable viral templates, these isolates were first tested with PCR primers (data not shown) which recognize all enteroviruses (Yang et al., Virus Res. 24:277-296 (1991)). Each of the primers listed in Table 3 were initially tested on a panel of viruses representing major NPEV groups (including Cav9, 12, 21, CBV1-6, echoviruses 4, 11, 30, EV71).

TABLE 3: PARTIAL SCREENING OF NEPVs WITH PCR PRIMERS FROM TABLE I

PRIMERS	SIZE	A9	A12	121	B1	B2	B3	B4	B5	B6	EC4	EC11	EC30	EV71
5S/6A*	101	+	-	-	+	+	+	+	+	+	+	+	+	-
6S/7A	134	+	-	-	+	+	+	+	+	+	+	+	-	-
7S/8A	143	-	-	-	-	-	+	+	+	-	-	-	-	-
7S/9A*	107	-	-	-	+	+	+	+	+	+	+	+	+	-
14S/11A*	130	-	-	-	+	+	+	+	+	+	+	+	+	-
24S/47A	77	+	-	-	+	+	+	-	+	+	-	-	+	-
24S/25A	98	+	-	-	-	+	-	-	-	-	-	-	-	-
24S/46A	98	+	-	-	+	+	+	-	-	+	-	-	-	-
34S/28A	86	-	-	+	-	-	-	-	-	-	-	-	-	-
34S/33A	89	-	-	-	+	-	-	+	+	+	-	-	-	-
34S/35A	104	-	-	-	-	-	-	-	-	+	-	+	-	-
34S/38A	101	-	-	-	-	-	-	-	-	-	-	+	-	-
34S/73A	98	+	-	-	-	-	-	-	-	-	-	+	-	-
36S/35A	80	-	-	-	-	-	-	-	-	-	-	+	-	-
39S/40A	71	-	+	-	-	-	-	-	-	-	-	-	-	+
39S/41A	62	-	-	-	-	-	-	-	-	-	-	-	-	+
51S/52A	83	-	-	-	+	+	+	+	+	+	+	+	+	-
55S/54A	140	-	-	-	-	-	-	-	-	-	-	-	-	+

Clarified lysates of infected cell cultures (1μ/reaction) were the source of templates for PCR reaction. After 30 amplification cycles DNA products were separated by electrophoresis on 12% polyacrylamide gels and visualized by ethidium bromide staining. The presence of a PCR product with the predicted size is indicated by a plus sigh (+) in the appropriate column for each isolate.

The results from screening this virus panel shown in Table 3 were used to determine how conserved the targeted amino acid sites were among this selected virus group. Sequence analysis of the PCR products amplified from temolated of several viruses (for example the 101 bp amplication product from the 5S/6A primer set) confirmed that the primers had primed specific amplification of the targeted nucleotide interval (data not shown). General patterns of reactivity can be determined from these results. In particular, primer sets 5S/6A, 7S/9A, 14S/11A, and 51S/52A were found to be broadly reactive with both CBVs and echoviruses. This amplification pattern agrees with the method, discussed above, that was used to select these particular primer sites. The amino acids for these four primer sets are shown to be conserved throughout the CBVs as well as the echoviruses in Table 1. The four antises primer sequences (i.e. primers 6A, 9A, 11A, and 52A) are not seen in the known CAV sequences shown in Table 1. This explains why there is no amplification when these primers are used on CAV isolates (with the noted exception of CAV9). Many of the remaining primers tested in Table 3 reacted with either a few virus isolates, or none at all. Primer sets that were either broadly reactive or selective in amplification were further tested using a larger panel of prototype NPEVs.

NPEV PCR primer pool.

A collection of 49 NPEVs was assembled for further PCR analysis. The bulk of NPEVs not found in this collection consists of CAVs, which grow very poorly in cell culture (Muir et al., J. Clin. Micro 31:31-38 (1993)); Rotbart, Human Enterovirus Infections, ASM, Washington D.C. (1995). This expanded virus panel was tested with PCR primer sets identified in Table 3. Primers 5S/6A, 7S/9A,

14S/11A and 51S/52A were found to be broadly reactive through all CBVs and most of the echoviruses (Table 4).

TABLE 4: NPEV PCR PRIMER POOL

EV	5S/6A	7S/9A	14S/11A	51S/52A	61S/68A	64S/65A	67S/1A	67S/8A
A3	-	-	-	-	+	-	+	-
A4	-	-	-	-	+	-	-	-
A5	-	-	-	-	+	-	-	-
A6	-	-	-	-	+	-	-	-
A8	-	-	-	-	+	+	+	-
A9	+	-	-	-	-	-	-	-
A10	-	-	-	-	+	+	-	-
A12	-	-	-	-	-	-	-	-
A14	-	-	-	-	-	+	-	-
A16	-	-	-	-	+	+	-	-
A21	-	-	-	-	-	-	-	-
A24	-	-	-	-	-	-	-	-
B1	+	+	+	+	-	-	-	-
B2	+	+	+	+	-	-	-	-
B3	+	+	+	+	-	-	-	-
B4	+	+	+	+	-	-	-	-
B5	+	+	+	+	-	-	-	-
B6	+	+	+	+	-	-	-	-
EC3	+	+	-	+	-	-	-	-
EC4	+	+	+	+	-	-	-	-
EC5	-	-	-	+	-	-	-	-
EC6	-	+	+	+	-	-	-	-
EC7	-	+	+	+	-	-	-	-
EC8	-	-	-	+	-	-	-	-
EC9	-	-	+	+	-	-	+	-
EC11	+	+	+	+	-	-	+	+
EC12	+	-	+	+	-	-	+	+
EC13	+	+	-	+	-	-	+	-
EC14	+	-	+	-	-	-	-	-
EC15	+	-	-	+	-	-	-	-
EC16	+	-	-	+	-	-	-	-
EC17	+	+	-	+	-	-	+	+
EC18	+	-	-	-	-	-	-	-
EC19	+	+	+	+	-	-	-	-
EC20	+	-	+	+	-	-	-	-
EC21	+	-	+	+	-	-	+	+
EC24	+	+	+	+	-	-	+	-
EC25	+	-	+	+	-	-	-	-
EC26	+	-	-	+	-	-	-	-
EC27	+	+	-	+	-	-	-	-
EC29	+	-	+	+	-	-	+	+

EV	5S/6A	7S/9A	14S/11A	51S/52A	61S/68A	64S/65A	67S/1A	67S/8A
EC30	+	+	+	+	-	-	-	-
EC31	+	-	-	+	-	-	+	+
EC32	+	-	+	+	-	-	-	-
EC33	+	-	+	+	-	-	-	-
EV68	-	-	-	-	-	-	-	-
EV69	+	-	-	+	-	-	+	-
EV70	-	-	-	-	+	-	-	-
EV71	-	-	-	-	+	+	-	-

Samples were analyzed as indicated in Table 3. The presence of a PCR product corresponding to the correct size for each different primer set is indicated with a plus sign. PCR reactions yielding either no product, or a product of incorrect size are indicated with a minus signs.

Primer set 67S/8A was shown to amplify selected echoviruses (EC11, 12, 17, 19, 21, 29, and 31) without reacting with CAVs and CBVs. The 67S/1A primer set reacted with a few CAVs (A3 & A8) and several echoviruses (EC9, 11, 12, 13, 17, 21, 24, 29, and 31). EV69 was also amplified by 67S/1A. The primer sets 61S/68A and 64S/65A amplified the predicted PCR product size with only CAVs (A3, A4, A5, A6, A8, A10, A16 for 61S/ 68A and A8, A10, A14, A16 for 64S/65A), as well as those viruses closely related to CAVs, i.e. EV70 and EV71. The 8 PCR primer sets identified in Table 4 can be used as a collection or "pool" of primer sets for rapidly performing a preliminary screen against suspected NPEV isolates.

A quick screen chart was assembled using these primers to aid in the screening of NPEVs (Table 5).

TABLE 5: QUICK SCREEN CHART FOR NPEV PRIMER POOL*

1	2	3	4	5	6	7	8	
-	-	-	-	-	-	-	-	=A12, A21, A24, EV68
-	-	-	-	-	+	-	-	=A14
-	-	-	-	+	-	+	-	=A3
-	-	-	-	+	-	-	-	=A4, A5, A6, EV70
-	-	-	-	+	+	+	-	=A8
+	-	-	-	-	-	-	-	=A9, EC18
-	-	-	-	+	+	-	-	=A10, A16, EV71
+	+	+	+	-	-	-	-	=B1, B2, B3, B4, B5, B6, EC4, EC30
+	+	+	+	-	-	+	+	=EC11
+	+	+	+	-	-	-	+	=EC19
+	+	+	+	-	-	+	-	=EC24
+	+	-	+	-	-	-	-	=EC3, EC27
+	+	-	+	-	-	+	+	=EC17
+	+	-	+	-	-	+	-	=EC13
+	-	+	-	-	-	-	-	=EC14
+	-	+	+	-	-	-	-	=EC20, EC25, EC32, EC33
+	-	+	+	-	-	+	+	=EC12, EC21, EC29
+	-	-	+	-	-	+	+	=EC31
+	-	-	+	-	-	-	-	=EC15, EC16, EC26
+	-	-	+	-	-	+	-	=EV69
-	+	+	+	-	-	-	-	=EC6, EC7
-	-	+	+	-	-	+	-	=EC9
-	-	-	+	-	-	-	-	=EC5, EC8

*Lane 1=5S/6A; 2=7S/9A; 3=14S/11A; 4=51S/52A; 5=61S/68A; 6=64S/65A; 7=67S/1A; 8=67S/8A.

Table 5 summaries Table 4. After analysis with the selected primer sets, samples can be quickly screened by comparing to Table 4. Table 4 provides the most likely serotype or group of serotypes which may be present in the sample. Further screening, using conventioanl micro-neutralization tests, can then be performed on only those suspected serotypes. This significantly reduces the number of micro-neutralization tests that need to be done, thus speeding up identification by eliminating unnecessary testing and conserving the limited amounts of Melnick antisera pools that are available.

This chart also shows that none of these primers reacted with CAV12, 21, 24 and did not detect EV68. Other PCR results shown in Table 1 specifically detect either CAV12 (39S/40A) or CAV21 (34S/28A). Primers, 63S/43A, specifically detect CAV24 (data not shown). Only one NPEV, EV68, was not amplified by PCR primers. Twelve of the isolates can be specifically identified using this primer pool (i.e. CAV3, 8,14, EC9, 11, 13,14, 17, 19, 24,31, and EV69). By first using these PCR primers to quickly screen virus isolates (within 1 day),

one can concentrate on performing micro-neutralization tests with only those monospecific antisera suggested by the PCR results.

Numerous modifications and variations in practice of the invention are expected to occur to those skilled in the art upon consideration of the foregoing detailed description of the invention. Consequently, such modifications and variations are intended to be included within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for designing and making a nucleic acid sequence effective for use as a degenerate PCR primer for amplification of a target nucleic acid sequence, the method comprising:

identifying uniquely conserved amino acid sequences;

synthesizing a nucleic acid sequence that corresponds to the identified uniquely conserved amino acid sequence; and

substituting the synthesized nucleic acid sequence with no more than about four pre-determined nucleotides at degenerate nucleotide positions, the pre-determined nucleotides being at least about 3 bases away from a 3' end of the synthesized nucleic acid, the synthesized nucleic acid sequence having no more than about seven degenerate positions and no more than about two adjacent pre-determined nucleotides.

2. The method of claim 1, wherein the degenerate PCR primer is about 17 to about 23 nucleotides in length.

3. The method of claim 2, wherein the degenerate PCR primer is about 20 nucleotides in length.

4. The method of claim 1, wherein the pre-determined nucleotide is selected from the group consisting of dI, dK and dP.

5. The method of claim 4, wherein the pre-determined nucleotide is dI.

6. A method for amplifying a target nucleic acid, the method comprising:

identifying uniquely conserved amino acid sequences;

synthesizing a nucleic acid sequence that corresponds to the identified uniquely conserved amino acid sequence;

substituting the synthesized nucleic acid sequence with no more than about four pre-determined nucleotides at degenerate nucleotide positions, the pre-

determined nucleotides being at least about 3 bases away from a 3' end of the synthesized nucleic acid, the synthesized nucleic acid sequence having no more than about seven degenerate positions and no more than about two adjacent pre-determined nucleotides;

adding the synthesized nucleic acid sequence to a PCR assay in an amount effective for amplifying any target nucleic acid present in the sample;

conducting PCR at a temperature effective for amplifying target nucleic acid; and

detecting the presence of amplified target nucleic acid.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A3

(11) International Publication Number: WO 99/53097

(43) International Publication Date: 21 October 1999 (21.10.99)

(21) International Application Number: PCT/US99/07513

(22) International Filing Date: 6 April 1999 (06.04.99)

(30) Priority Data:
60/081,944 15 April 1998 (15.04.98) US

(71) Applicant *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES CENTERS FOR DISEASE CONTROL AND PREVENTION [US/US]; 255 East Paces Ferry Road, N.E. Room 500, Atlanta, GA 30333 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: KILPATRICK, David, R. [US/US]; 1095 Fulton Court, Norcross, GA 30093 (US).

(74) Agents: SAMPLES, Kenneth, H. et al.; Fitch, Even, Tabin & Flannery, Room 1600, 120 South LaSalle, Chicago, IL 60603 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
18 November 1999 (18.11.99)

(54) Title: METHOD FOR DEVELOPING DEGENERATE PCR PRIMERS

(57) Abstract

The present invention provides a method for designing and making degenerate primers for use in amplification assays. Using the present method, degenerate primers for the amplification and subsequent detection of virtually all genes that encode an amino acid sequence can be obtained. The degenerate primers are effective for detection of any gene which lies within a coding region that results in the production of a protein. Examples of genes that can be detected include those where the sequence of the specific target gene is known or unknown and where the amino acid sequence encoded for by the gene is structural, nonstructural, or enzymatic. The method provides highly specific primers which are effective for substantial amplification of a target sequence even where the target nucleic acid sequence is unknown.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

INTERNATIONAL SEARCH REPORT

Int[redacted]al Application No
P S 99/07513

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 02704 A (US GOVERNMENT) 26 January 1995 (1995-01-26) examples ---	1-6
X	PATIL R V ET AL: "PCR AMPLIFICATION OF AN ESCHERICHIA COLI GENE USING MIXED PRIMERS CONTAINING DEOXYINOSINE AT AMBIGUOUS POSITIONS IN DEGENERATE AMINO ACID CODONS" NUCLEIC ACIDS RESEARCH, vol. 18, no. 10, 25 May 1990 (1990-05-25), page 3020 XP000310539 ISSN: 0305-1048 cited in the application the whole document --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1999	12/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Molina Galan, E

1

Form PCT/ISA/210 (second sheet) (July 1992)

[redacted]onal Application No
[redacted]/US 99/07513

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	FORDHAM ET AL.: "Synthesis of saporin gene probes from partial sequence data" MOL. GEN. GENET., vol. 221, 1990, pages 134-138, XP002116639 cited in the application figure 1	1-6
X	EP 0 712 933 A (NEW ENGLAND BIOLABS INC) 22 May 1996 (1996-05-22) the whole document	1-6
X	DE 43 38 817 A (MAX DELBRUECK CT FUER MOLEKULA) 14 June 1995 (1995-06-14) the whole document	1-6
X	WO 94 03644 A (CAMBRIDGE NEUROSCIENCE INC) 17 February 1994 (1994-02-17) the whole document	1-6
X	US 5 268 465 A (BREDT DAVID S ET AL) 7 December 1993 (1993-12-07) the whole document	1-6

1

INTERNATIONAL SEARCH REPORT

[redacted] on patent family members

International Application No
PCT/US 99/07513

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9502704 A	26-01-1995	US 5585477 A	17-12-1996
		AU 693465 B	02-07-1998
		AU 7332494 A	13-02-1995
		EP 0789782 A	20-08-1997
		JP 9500530 T	21-01-1997
		US 5691134 A	25-11-1997
EP 0712933 A	22-05-1996	US 5543308 A	06-08-1996
		JP 8224088 A	03-09-1996
DE 4338817 A	14-06-1995	NONE	
WO 9403644 A	17-02-1994	AU 693767 B	09-07-1998
		AU 5002693 A	03-03-1994
		CA 2142185 A	17-02-1994
		EP 0656069 A	07-06-1995
		JP 8500246 T	16-01-1996
		US 5876973 A	02-03-1999
		ZA 9305801 A	19-04-1994
US 5268465 A	07-12-1993	NONE	